PB-230 376

BIOCHEMICAL EFFECTS OF SIMULATED AIR POLLUTANTS

Frances L. Estes

Gulf South Research Institute
Baton Rouge, Louisiana

20 July 1971

DISTRIBUTED BY:

NTIS

National Technical Information Service
U. S. DEPARTMENT OF COMMERCE
5285 Port Royal Road, Springfield Va. 22151

PB 230 376

Reproduced by
NATIONAL TECHNICAL
INFORMATION SERVICE
U S Department of Commerce
Springfield VA 22151

SUMMARY

AP 1153

Frances L. Estes

Gulf South Research Institute
Baton Rouge, La.

BIOCHEMICAL EFFECTS OF SIMULATED AIR POLLUTANTS

Final Report

Previous work has indicated the multiplicity of factors involved in the valid determination of the dose-response relationship of photochemical reaction products and biological or individual biochemical systems. The work reported herein was directed to the further assessment and control of these factors.

For the over-all evaluation of the response of tissues, the activity of particular dehydrogenase system may be most revealing. Exploration of the response of lung tissue to hydrogen peroxide indicated that the breakdown of the hydrogen peroxide by the catalase in the tissue provided an apparent protective effect, that exposure to light (3400Å) increased the inhibition of lactic dehydrogenase by H_2O_2 and that the presence of the malic dehydrogenase system complicates the determination of the lactic dehydrogenase activity by change of absorbance of the coenzyme. The method which has been developed for the quantitative extraction of alpha substituted short chain fatty acids from tissues permits the direct determination of the activities of these enzymes as well as the initial concentration of the substrates and products by gas chromatography.

The possibility that the diverse responses of biological materials to photochemical reaction products results from the differents in free radical formation during photolysis is consistent with the nature of the reactions of biochemical systems. Preliminary observations of the isomeric products of oxidation of limonene indicates that the distribution of the products is a function of the relative concentrations of the butene-nitrogen dioxide mixture and that singlet oxygen was present in the photochemical reaction products of some of the mixtures. Further exploration of these observations should indicate the contribution of free radicals and/or singlet oxygen to biological responses, and in turn assist in the development of valid dose-response relationships.

July 20, 1971

EXPLORATION OF THE FACTORS INVOLVED IN THE EVALUATION OF EFFECTS OF AIR POLLUTANT COMPONENTS ON THE DEHYDROGENASES IN TISSUES

INTRODUCTION

Since tissues as homogenates, slices or *in situ* are composed of numerous biochemical systems interacting in a sequential manner (i.e. one reaction provides the substrate for the second reaction, etc.), and of systems which may compete for specific substrates, it is necessary to know the factor which may be involved in the apparent response of the tissue to any given pollutant. Thus in effect, one is saying that the response of a tissue is more than a simple algebraic sum of the component parts.

For an over-all evaluation of the response of a tissue, the activity of particular dehydrogenase systems may be most revealing. Specifically, the key enzymes of various metabolic systems are lactic dehydrogenase, malic dehydrogenase, and glucose-6-phosphate dehydrogenase. If we are to determine the effects of air pollution on tissues, it is essential that our method of analyses be free of artifact and that all contributive variables be accounted for.

The following experiments have indicated specific precautions in the experimental procedures involving the effect of air pollutants on lung tissues, as well as serving to train personnel for more extensive procedures involved in subsequent evaluations of the responses of lung tissues to mixed pollutants vapor and gases.

METHODS

Nitrogen content of the tissues use determined by the Dumas method using a Colman Nitrogen Analyzer. Enzyme activities were determined by the rate of change of absorbance of the respective coenzyme at 340 millimicrons using a Beckman DK-2.

For the exposures the appropriate concentration of H_2O_2 was contained in the appropriate buffer and added to the tissue homogenate. The controls were prepared by the addition of an equivalent amount of buffer to the tissue homogenates. Incubations were at constant temperature with continual mixing by mechanical oscillation for the times indicated.

Appropriate aliquots of the incubated tissue were used for the determination of enzymatic activity. The Lactic Dehydrogenase system contained 2 micromoles of sodium pyruvate, 0.4 micromoles of NaDPNH, and 200 micromoles phosphate buffer adjusted to pH of 7.4 with an appropriate concentration of enzyme or tissue to make the total volume of the sample 3 ml. The Malic Dehydrogenase system contained 2 micromoles of oxalacetic acid, 0.4 micromoles of Na DPNH, and 200 micromoles of phosphate buffer adjusted to pH 7.4 with an appropriate concentration of enzyme or tissue; total volume of the system to 3 ml. The Glucose-6-phosphate Dehydrogenase system contained 4 micromoles glucose-6-phosphate, 0.15 micromoles TPN, 10 micromoles glycylglycine buffered to pH 7.5, and 20 micromoles $MgCl_2 \cdot 6\ H_2C$ with an appropriate concentration of enzyme or tissue; total volume of the system to 3 ml.

EXPERIMENTAL

1. Determination of variable involved in determination of effects of hydrogen peroxide on lung tissue.

Using change of absorbance of the coenzyme for the determination of lactic dehydrogenase activity, the response of the assay system to various concentration of the enzyme is shown in Table I. Similarly, in Table II is shown the lactic dehydrogenase activity of lung tissue homogenates. In each instance it is evident that at the higher concentrations of enzyme less activity was observed. Thus, for subsequent experiments with these enzyme suspensions the lower concentrations of enzyme were used in the preparation of the assay system. Because of the variation of lactic dehydrogenase activity from one preparation to another, the amount of the preparation to be used for its assay must be evaluated on the preparation in question.

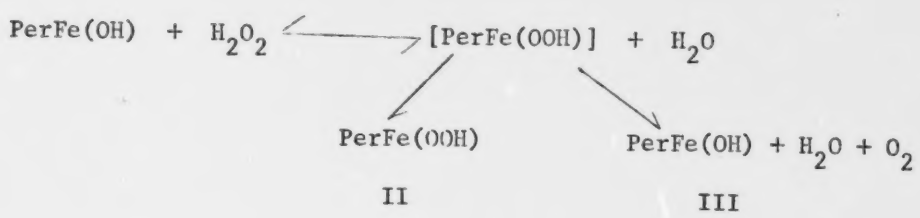
In passing it may be noted that for comparable change in absorbance the lung tissue contains approximately one hundred times more nitrogen. This tissue nitrogen may not be regarded as extraneous or unreactive and includes other enzyme systems.

For the evaluation of the effect of an extraneous material on an enzyme system, it is necessary to determine concentration of the material and exposure time. One such experiment is shown in Table III. It is to be noted that one hour incubation at 24°C leads to a 46.3% activity of the

controls, hence loss of activity was based on the unincubated, unexposed enzyme. Likewise, it is to be noted that doubling the concentration of the hydrogen peroxide leads to a factor of 4 in the loss of enzymatic activity.

Exploration of the factors involved included examination of the effect of H_2O_2 on the absorbance of the coenzyme. The absorption of a solution of 2.256×10^{-3} M DPNH containing 8.8 millimoles of H_2O_2 gave .0689 reduction in the absorbance of the DPNH at the end of 5 minutes. The absorbance of H_2O_2 at 248 millimicrons was unchanged. For lactic dehydrogenase solution containing 0.044 millimoles of H_2O_2 , the possible change of the absorbance of the enzyme at 280 millimicrons was observed by the absorbance of the H_2O_2. The 248 millimicrons absorbance of H_2O_2 was unchanged over a period of 30 minutes.

However, when dog lung tissue homogenate was used in place of the purified lactic dehydrogenase, H_2O_2 was consumed. A zero time scan of the homogenate in the presence of H_2O_2 the 248 millimicrons absorbance of the H_2O_2 was pronounced. After 30 minutes the 248 millimicrons absorbance of H_2O_2 was still evident but markedly reduced. These observations are accounted for by the presence of peroxidase activity in the lung tissues. The reactions involved may be seen in the following equation:



when the peroxidase is represented as Per(Fe)OH, the initial reactive enzyme product indicated as I which can go to the inactive product II or on a further with H_2O_2 return to original enzyme (III). Thus, the stoichiometry becomes distruction of 2 H_2O_2 molecules per peroxidase molecule. At this time the specific activities of the peroxidases was not determined since it was very apparent that peroxidase activity could explain the need for extremely high concentrations of H_2O_2 in the incubation mixture of lung tissue homogenates to observe any effect of the H_2O_2 on the tissue homogenates. Thus, in evaluation of the effects of pollution mixtures containing peroxides or ozone on tissue, peroxidase as well as dehydrogenase activities must be determined.

During the determination of absorbance of the coenzyme, the enzyme mixtures are exposed to ultraviolet light (340 millimicrons). The wavelength and time of exposure are identical and as indicated above, the effect of H_2O_2 on the absorbance of the coenzyme is negligible for short scan times. (Activities determined on change during the first two minutes, while the perceptible effect of H_2O_2 required 5 minutes). This, however, does not indicate the possible increase of the effect of H_2O_2 by exposure of the incubation system to light. Two such experiments are shown in

Table IV. These experiments serve to indicate that at constant concentration of the H_2O_2, increased exposure times lead to increased inhibition. Likewise, it appears that exposure to light enhances the inhibition of lactic dehydrogenase by H_2O_2.

Since the dehydrogenases of concern involve similar coenzymes and methods of determination, it is necessary to know the extent of response of a specific dehydrogenase to other substrates. In Table V the response of purified lactic dehydrogenase (LDH) to its normal pyruvate substrate and to oxaloacetic acid substrate of the malic dehydrogenase (MDH) system. The fact that without any attempt to optomize conditions, purified LDH contains apparent MDH activity, indicates that this undoubtedly occurs in whole tissue preparations. Thus, in evaluating relative effect of an inhibitor, this indirect method of determining activity could be grossly misleading. For definitive experiment the disappearance of the substrate and/or formation of product must be determined.

2. Examination of malic dehydrogenase activity.

Previous observation had indicated that lung tissue contains considerable malic dehydrogenase (MDH) activity. In preparation for using this system as a means of evaluating the effect of air pollutant mixtures on MDH activity, the following explorations were made. Table VI shows the effect of incubation time on the MDH activity of a dog lung homogenate. As is frequently observed, incubation with mixing increases the activity of the system. In this case the 13.6% increase in activity of the system again points to the importance of using incubated controls as standard and serves to emphasize the loss of activity of the controls with prolonged (i.e. 60 minutes) incubation.

In another experiment using 20 minute incubation periods, a slightly lower quantity of homogenate shows 11.1% loss of activity in 20 minutes of incubation (Table VII). Of more importance was the observation that after 20 minutes of incubation, the presence of 2.1 millimoles of H_2O_2 led to complete inactivation of the MDH system, whereas in the presence of 0.35 millimoles of H_2O_2 or 1/6 the concentration, 62.5% of the MDH activity remained. A similar experiment is shown in Table VIII. Comparison of the data in these experiments serve to indicate that the absolute amount of an contaminant added is of less importance than the relative amount with respect to the tissue involved. This observation is characteristic of the type of data dealing with living systems

and serves to emphasize that these responses are dependent on concentrations of each component present. Not shown in the tables is the indication that in general, 20 to 30 minutes incubation time would be most revealing and that our limits of concentration of H_2O_2 are within the range shown in Table VII. Other experiments served to indicate that more definitive information could be obtained with lower H_2O_2 concentrations and longer incubation time. These experiments have not been corrected for peroxidase activity of the tissue. It has been assumed that in working with a single homogenate from an individual tissue that the peroxidase activity was constant.

From the information currently available, it appears that at lower concentrations of air pollutants exposure times of up to 60 minutes can provide valid observations. It should be noted that for the experiments thus far, the response of the MDH activity has not been corrected for LDH activity of the system. Likewise, it should be noted that as in LDH activity the presence of peroxidase activity in the tissue provide an apparent protective effect.

3. Examination of glucose-6-phosphate dehydrogenase activity

Initial examination of dog lung serves to indicate that there is considerable Glucose-6-phosphate dehydrogenase activity in dog lung. The data in Table IX suggests that for the usual concentration of the substrate (G-6-PO_4) the tissue nitrogen activity should be in about the 100 microgram range, or over (i.e. the activity on a nitrogen basis is comparable in this range).

Preliminary explorations appear to indicate that there is considerably more glucose-6-phosphate dehydrogenase activity in puppy than in dog lung. These differences in enzymatic activity as well as the sensitivity of the substrate system have served to complicate the evaluation of this system to an inhibitor (H_2O_2). The latter difficulties have in part been negated by the selection and control of buffer system as well as preparation of the substrate (glucose-6-phosphate).

SUMMARY

Lactic dehydrogenase, malic dehydrogenase and glucose-6-phosphate dehydrogenase activities in dog lung homogenates have been examined in the presence and absence of hydrogen peroxidase as inhibitor. It was observed that considerable lactic dehydrogenase activity is lost on storage of the tissue and that even purified lactic dehydrogenase shows considerable malic dehydrogenase activity as judged by the conversion of the malic dehydrogenase substrate (oxaloacetic acid). For each of the dehydrogenase systems the high concentrations of H_2O_2 required for inhibition is a function of the peroxidase activity of the tissue destroying the H_2O_2.

These observations further emphasize the precaution and controls required for valid evaluation of the response of a single tissue to an air pollutant mixture.

TABLE I

Response of Purified Lactic Dehydrogenase (Sigma) to Pyruvate Substrate System.

A.

Nitrogen Content*		Activity	
Second Dilution Ratio	Micromoles	DPNH micromoles/min	Micromoles/mg Nitrogen/min
1:25	0.0882	7.	87.5
1:12.5	0.1599	9.2	57.5
1:10	0.200	15.3	76.5
1:5	0.400	40.2	100.5
1:3.3	0.600	53.9	89.8

*Calculated from reported content assuming 15% Nitrogen present. Preparation of enzyme system: To .04 ml of enzyme suspension was added 0.01 M NaCl to a volume of 25 ml. The solution was subsequently diluted as indicated so that 1 ml of the resulting suspension could be used for assay.

B.

Ml, LDH	Micromoles Nitrogen	Micromoles DPNH/min	Micromoles DPNH/min/micro-moles Nitrogen
.100	.20	71.0	355
.050	.10	42.8	428
.025	.05	21.6	432
.010	.02	11.6	580

Enzyme solution prepared as above from new lot and not separately diluted. Volumes of enzyme solution as indicated.

TABLE II

Response of Dog Lung to Lactic Dehydrogenase
Test System - Pyruvate Substrate

		Activity	
Ml. Lung Homogenate	Micrograms Tissue N*/ml	DPNH Micromoles	Micromoles/mg tissue Nitrogen/min
0.1	75.83	0.4148	5.470
0.08	60.70	0.3608	5.944
0.06	45.53	0.3055	6.799
0.04	30.35	0.1925	6.343
0.02	15.18	0.1170	7.708

*As determined with Colman nitrogen analyzer
0.7583 mg N/ml of homogenate.

TABLE III

Effect of Concentration of H_2O_2 on
Lactic Dehydrogenase Activity (Sigma)

	Enzyme N Micrograms	H_2O_2 millimoles incubation	Activity: NADH Micromoles/min	Activity: Micromoles/min/micrograms N	% Activity
	0.42	0	2.5	5.11	100
	0.42	.088	0.2	0.47	8
	0.42	.044	0.8	1.90	32
Zero Time	0.42	0	5.4	12.85	

Incubation mixture: Original enzyme suspension (.08 ml) diluted with 10 ml of 0.01N NaCl. Then 2 ml of that dilution further diluted to 10 ml. In appropriate flasks, 0.14 ml of final enzyme dilution taken to a total volume of 2 ml. with NaCl and H_2O_2 in NaCl. The H_2O_2 added was 1 ml of .088M and 0.5 ml of 0.088M. 1 ml each used for analysis after 1 hour incubation except for zero time sample analyzed immediately.

TABLE IV

Effect of Light on the Response of
Lactic Dehydrogenase (Sigma) to Hydrogen Peroxide

Incubation Mixture	Enzyme N Micrograms	H_2O_2 in incubation mixture millimoles	Exposure Time, min. With Light	Exposure Time, min. Without Light	Activity Micromoles DPNH/ Min.
A	0.33	0.880	8	8	4.1
	0.33				8.8
	0.33				1.1
	0.33		18	18	7.0
B	0.38	0.528	1	1	17.6
	0.38				17.7
	0.38		7	7	12.5
	0.38				16.3
	0.38		14	14	12.9
	0.38				8.9

Incubation Mixture A: Original enzyme suspension (0.02 ml) diluted to 25 ml with 0.01 N NaCl. In appropriate flasks one protected and one not protected from light 1 ml enzyme solution and 2 ml H_2O_2 (0.440M) were combined. Incubation mixture contained 3 micrograms enzyme N and 0.880 millimoles H_2O_2 in total volume of 3 ml.
Incubation Mixture B: Original enzyme suspension (0.01 ml) diluted to 10 ml with 0.01 N NaCl. In appropriate flask 1.0 ml enzyme preparation and 2 ml of H_2O_2 (0.264M) Incubation mixture contained 3.8 micrograms enzyme N and 0.528 millimoles H_2O_2 in a total volume of 3 ml.
In each exposure 0.3 ml used analyses.

TABLE V

Response of Lactic Dehydrogenase (Sigma) to
Oxalacetic Acid Substrate of Malic Dehydrogenase

	Activity
	Micromoles DPNH/Min
Pyruvate Substrate	56.8
Oxalacetic Acid Substrate	25.7

TABLE VI

Stability of Malic Dehydrogenase of Dog Lung

Conditions	Tissue N Microgram	Activity Micormoles DPNH/min	Activity %
No incubation	46.5	4.4	100
30 min at 24°	46.5	5.0	113.6
60 min at 24°	46.5	4.2	95.5

Tissue Preparation: 0.12 ml of original to 2 ml with 0.01 N NaCl 46.5 microgram N/ml

TABLE VII

The Effect of H_2O_2 on Malic Dehydrogenase Activity of Dog Lung

Conditions	Tissue N in Assay Micrograms	H_2O_2 in incubation Millimoles	Activity Micromoles DPNH/min	% Activity Remaining
No incubation	49.6	0	3.6	
Incubation	49.6	0	3.2	100
Incubation	49.6	2.11	0	0
Incubation	49.6	0.35	2.0	62.5

Incubation: 0.1 ml lung homogenate to 2.5 ml with 0.01 N NaCl for each sample. For the H_2O_2 sample 2.11 micromoles H_2O_2 in NaCl and 0.352 micromoles. All incubations 20 min

TABLE VIII

Effect of Hydrogen Peroxide
on Malic Dehydrogenase Activity
in Lung Tissue

Conditions	Tissue N In Assay Microgram	H_2O_2 in Incubation Micromoles	Activity	% Activity Remaining
Incubated	46.5	0	3.3	100
Incubated	46.5	0.53	2.0	60.6

TABLE IX

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY

Dog Lung
(Glycylglycine Buffer)

Homogenate (ml)	Glucose-6-Phosphate (ml)	Protein Microgram Nitrogen	Activity TPN micromoles/min	Micromoles TPN/microgram Nitrogen/min
.00	.2	0.0	0.0	------
.05	.2	38.78	0.3	.0077
.10	.2	77.56	1.2	.0155
.30	.2	232.68	4.1	.0176
.30	.4	232.68	5.8	.0249

RECOVERY OF LACTIC, PYRUVIC ACIDS AND SOME KREBS CYCLE COMPONENTS FROM TISSUES

(Preliminary Manuscript)

Frances L. Estes

Introduction

Numerous methods have been developed for the determination of various mono- and di-basic acids which occur in biological tissues. As might be expected the short chain alpha substituted acids have presented problems in their analysis and a variety of procedures have been developed. Thus Teaford and Kaplan[1] reported a gas chromatographic method for the determination of blood lactate by oxidation of it with ceric sulfate to acetaldehyde and analysis of the acetaldehyde on a column of SE-30 on Anakrom AB. This procedure does not, however, provide for the determination of pyruvate. The use of lactate and pyruvate as an index of severity of circulatory shock illustrates the importance of both determinations.

The observation that the usual indirect method of determination of the activity of dehydrogenases by the rate of change of absorbance of the coenzyme may not be valid in the presence of inhibitors[2] requires that the disappearance of the substrate and/or formation of the product be determined by direct means. In addition to the examination of abnormal dehydrogenase reactions, the interrelationship of sequential or competing reactions as well as determination of steric configuration require direct determination and identification of the products and substrate involved.

20<

Lactic and pyruvic acids as well as Krebs Cycle components may be determined in small volumes and at low concentrations by gas chromatographic analysis of their methyl esters.[3] From simple mixtures uncomplicated by extraneous salts proteins or other materials, good recoveries were obtained for lactic, pyruvic, malonic, succinic and malic acids. The usefulness of the analytical procedure for biological materials requires recovery of the acids from complex mixtures.

21<

Methods

For chromatography an 8-foot "U"-shaped column (10 mm I.D.) packed with 15% diethylene-glycol succinate (DSGE) on 80-100 mesh Gas-Chrom-P (Applied Science Laboratories) was used in a Barber-Colman Series 5000 gas chromatography with an Argon Strontium-90 Detector. Peak areas were measured by the attachment of a Model CRS-1 chromatography Readout System (Infotronics Co.)

Results

Application to Products and Substrates of Dehydrogenase Reactions.

Initial interest in this area developed from observations of the inhibition of dehydrogenase systems by air pollutant materials. Since a means for determining the reactants involved had been developed[3], the lactic dehydrogenase reaction was selected as a point of departure. For this work isolated lactic dehydrogenase (Sigma) was used. The reaction system with a pyruvate substrate contained 1 millimole of sodium pyruvate, 0.2 millimoles DPNH, 0.34 millimoles phosphate buffer (pH 7.5) and 0.1 ml enzyme suspension (0.8, eg protein).

It was evident that the enzyme must be"killed" quickly and by such means that the pyruvate substrate or lactate produced could be recovered simply and completely. The use of heat or acidification fulfilled the first but not the second condition. These and other procedures were complicated by the volatility of pyruvic acid or what appeared to be binding of the acids to the enzyme.

The selected procedure was the use of ethyl ether. At the required time the reaction was killed by the addition of 3 volumes of ice cold ethyl ether and the mixture rapidly evaporated under reduced pressure. The residue was extracted five times with 10 ml of acidified ether. The combined ether extracts were treated with excess diazomethane in ether (30 ml). After the reaction was completed, the excess diazomethan- and ether were removed under reduced pressure. The oily residue was taken up in ether and applied to the column.

Of the initial 10 micromoles of pyruvate present in the complete reaction system, 85% was recovered at zero time. Since better recoveries (95%) were achieved by cooling the reaction before the addition of the enzyme, and by simultaneous addition of the cold ether and the enzyme

solution, it became apparent that the recovery was dependant on manipulation time. The subsequent disappearance of the pyruvate substrate was linear with time and indicated enzymatic activity in agreement with that obtained from the change of absorbance of the coenzyme when determined by spectroscopic or flourosence procedures. (Figure 1).

In the initial experiments, the recoveries of the lactate produced were greater than that expected from the pyruvate disappearance or the change of absorbance of the coenzyme. Considerable time and effort was devoted to alleviation of this problem. The diazomethane was prepared methanol free and methanol free ether was used. Although these precautions are useful in eliminating excessive methanol peaks, which may be helpful under particular situations, the difficulty of the lactate recovery was solved by replacing the column with fresh packing material. Since the initial column had been in use for several years and utilized for a variety of material, it was difficult to determine the cause of the change in the column packing material.

In subsequent experiment with uninhibited systems, the rate of lactate formation parallel that of the pyruvate consumed. Likewise, using a lactate system, the pyruvate formed parallel that of the lactate consumed. Further evaluation of the accuracy of the method will be obtained from the control required in the application of the procedure to inhibited systems.

Preliminary experiments with a pyruvate system and formaldehyde as the inhibitor indicate that the rate of disappearance of the coenzyme is more rapid than that of the rate of consumption of the pyruvate or appearance of the lactate.

Application to Blood.

An attempt to directly methylate blood serum by the diazomethane

procedure was unsuccessful. Hence, various pre-treatments of blood were examined. They included the Folin-Wu, perchoric acid, and metaphosphoric acid precipitations of the protein. The perchloric acid precipitation which appears to be the most frequently used for lactate, gave poor recoveries. It is suspected that the precipitation of the potassium chlorate included some lactate or pyruvate. For us the meta-phosphoric acid was preferable for both rabbit and human serum.

The procedure was as follows: To 2 ml of blood serum, 6 ml of 5% meta-phosphoric acid was added. After the mixture was allowed to stand for 15 minutes, the precipitate was removed by centrifugation in a clinical centrifuge. The clear filtrate was removed and immediately neutralized with NaOH. For esterification three ml of neutralized filtrate was transferred to a round bottomed flask and acidified with 4 ml of 6 N HCl (pH 1.0). The acidified a mixture was immediately extracted 5 times with 10 ml of ether. The the combined ether extracts, 30 ml of diazomethane in ether was added and the mixture allowed to react at room temperature for 15 minutes. The ether and unreacted diazomethane were removed by evaporation on a flask evaporator (30°C). The reduced droplet was dissolved in 1 ml of ether. For analysis of these samples, appropriate amounts (1 - 2 μl) were injected on the chromatographic column.

In the development of this procedure, the previous difficulties led to an emphasis on the recovery and analysis of lactate. For a group of samples of human serum obtained from a clinical laboratory, the pyruvate concentration varied more widely than did the lactate concentration. This observation prompted care in the addition of the metaphosphoric acid to the serum so that there was no overheating as well as the neutralization of the filtrate. With these precautions, added sodium lactate and/or sodium pyruvate to the blood serum were completely accounted for and it is probable that the observed variations of pyruvate concentration represented actual variation in the samples. It should be noted that on storage of the serum samples, decreasing amounts of pyruvate were found.

Although further determinations are required for the evaluation of the accuracy of the recovery procedure for blood, it is apparent that freshly drawn blood should be used.

Since blood sera proveded a complex mixture, it was used to evaluate possible problems of recovery of other acids. No difficulties in the recovery and esterification of malonic, succinic, or malic acid were encountered.

Although for purposes of identification, determination of these diverse methyl esters by Gas Chromatography may be programmed; quantification requires isothermal operation. Column temperatures of 85°C and flask heater temperatures of not more than 250°C were used for the determination of methyl pyruvate and methyl lactate. For Malonate succinate and malate, column temperatures of 125°C were used. This method of operation provided for more rapid analysis without undue sacrifice of recovery of materials from the columns.

Working Conclusion

The use of ether extraction, acidification and methylation provides for the recovery and the determination of pyruvate, lactate, succinate malonate and malate from biological tissue systems. Precise evaluation of the accuracy of the procedures involved and the application of the methods to inhibited enzyme systems was thwarted by difficulties with the feed back of electrical current from other parts of the building. With the relocation of the laboratory, the problem has been negated and further evaluations are being undertaken.

REFERENCES

1. Teaford and Kaplan. Clin. Chem. Acta 15 133, 1967.

2. Estes and Pan. Arch. Environ. Health 10 207, 1965.

3. Estes and Bachmann. Anal. Chem. 38 1178, 1966.

28<

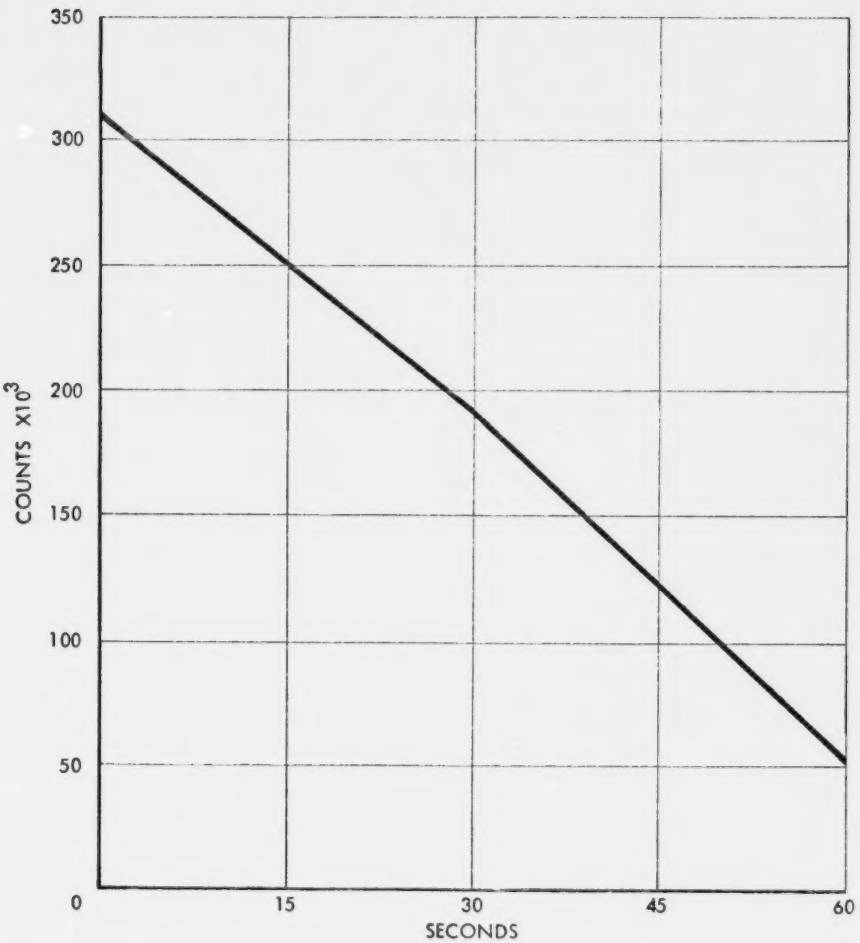


Fig. 1 PYRUVATE DISAPPEARANCE WITH REACTION TIME

EXPLORATION OF AIR POLLUTANT MIXTURES FOR THE PRESENCE OF SINGLET OXYGEN

Preliminary Manuscript
Frances L. Estes

Although multiplicity of physical and biological effects have been attributed to air pollutants mixtures, the specific components of the air pollutant mixtures which may be responsible for a number of detrimental effects have not been identified. In 1967 Khan, et al, showed theoretically that the formation of singlet oxygen could account for the observed distribution of products during photochemical smog formation. Excited singlet state molecular oxygen is a reactive form of oxygen containing some 22 calories more energy than ordinary (triplet) oxygen. The singlet state molecular oxygen is a transient, hard to detect species. Thus, analogous to enzymes, singlet oxygen is known by what it does, specifically its ability to form unstable peroxides with a variety of chemical compounds. Among the potential sources of singlet oxygen in polluted atmospheres are: the direct excitation of ordinary (triplet) oxygen, energy transfer from light absorbing pollutants, photolysis of ozone, and decomposition of previously formed peroxyacetylnitrate (PAN).

As yet there has been no direct evidence that singlet oxygen does indeed occur as a product of photochemical air pollution. Quite apart from the substantiation of the theory, such a demonstration could explain the diverse biological responses to the photochemical reaction

products and provide impetus for the delemination of those pollutants most important to the formation of singlet oxygen. Using the oxidational products of limonene as a detector, synthetic air pollutant mixtures produced by the irradiation of various concentrations of butene-nitrogen dioxide mixtures in air were examined for the presence of singlet oxygen.

METHODS

The limonene was purified by preparative gas chromatograph. The purified material was free of carvone and gave a single chromatographic peak. After the extraction and concentration procedure used for the treated samples, chromatograph peaks following limonene accounted for 8% of the material eluted.

For the chemical oxidation 8.6 grams of d-limonene was dissolved in 150 ml of a 1:1 mixture of butanol and methanol. The The solution was cooled to less than 10°C and 30 ml of H_2O_2 added. With stirring 200 ml of 30% NaOCl was added dropwise over a period of two hours. For reduction 1.5 grams of $NaBH_4$ was added to the cold reaction flask and allowed to stir overnight. The following morning the solution was extracted three times with 200 ml pentane. The pentane solution,washed with Na_2SO_4 solution,was dried over night with anhydrous Na_2SO_4. The pentane solution was evaporated on a rotary evaporator. The residual solution contained limonene and the oxidational product.

For treatment with gases 2.8 ml of d-limonene in 50 ml of a 1:1 mixture of butanol and methanol were used. The flask containing the limonene solution was provided with an inlet and outlet tube, and

suspended in an ice bath. The inlet tube was connected directly to the gas irradiation flask but protected from the associated light. The desired concentration of nitrogen dioxide and/or butene in air were obtained by serial dilution with a previously described apparatus[1]. and led directly into the irradiation chamber. After exposure to the irradiated gas stream, the limonene solution was reduced, extracted, dried and evaporated as described above.

Analyses were made on a Barber-Colman Gas Chromatograph with hydrogen flame detector, using a 10 foot 1/4 inch pack column containing 15% diethylene glycol succinate on 80-100 mesh Gas-Chrom P at 150°C with a carrier gas flow rate of 65 ml/min. Peak areas were measured by the attached Chromatography Readout System (Infotronics Co.).

RESULTS

The experimental approach was based on the extensive work of Foote and Associates.[2,3] The specific observation of interest was the distribution of the isomeric products resulting from the oxidation of (+) limonene by different methods.[4] Thus of the six isomeric alcohols shown in Table I, the ones identified as III and VI are formed by way of singlet oxygen but not from radical oxidation.

The first column of data in Table II shows the gas chromatographic pattern of limonene itself after extraction and concentration. The four peaks following limonene represent unidentified contaminants. Although the components previous to limonene have not been shown, the total count has been included to indicate the comparative concentrations of the samples in the solvent. The four peaks following limonene represent unidentified contaminants which had not been removed in the purification. The removal of contaminants of longer retention times (e.g. carvone) was necessary to the analysis of the isomeric alcohols.

Initially it was hoped that chemical oxidation of limonene by the use of hydrogen peroxide and hypochlorite solution would give a complete spectra of the alcohols. Unfortunately, limonene is not an avid acceptor. With the addition of 2,6-di(t-bytyl) phenol as radical inhibitor, the chromatographic analysis was obscured by the presence of the inhibitor which carried through the extraction.

The data shown is that from the treatment of limonene with hydrogen peroxide and hypochlorite solution in the absence of a radical inhibitor. Not all of the expected products were formed, and may have resulted from the solubility difficulties involved in treating limonene with aqueous hypochlorite solution even though the mixed solvent system was used.

For the examination of the oxidational products resulting from treatment with gaseous systems shown in Table II, the limonene (0.43 ml) was dissolved in a 15 ml of a 1:1 mixture of methanol and Butanol and exposed for various periods of time thus: un-irradiated air 6 hours, irradiated air 12 hours, and air containing 0.86 ppm nitrogen dioxide 6 hours. From these experiments it appeared that 6 hours exposure was sufficient to form the oxidational products in amounts sufficient for detection. Although the exposure times are not comparable, it should be noted that while unirradiated air produced oxidational products that would be formed by radical oxidation, with irradiated air, 80% of the oxidational product was the alcohol labeled III which is formed from singlet oxygen oxidation. On the addition of 0.86 ppm nitrogen dioxide after 6 hours exposure, the products formed by way of singlet oxygen were both present, III and VI representing 17 and 37 % respectively of the products formed.

In order to have more uniform exposure of the limonene solution, a round bottomed (50 ml) flask was used and the amount of limonene alcohol mixture was doubled, i.e. 0.86 ml limonene in 30 ml of the 1:1 methanol-t-butanol mixture. The distribution of products after treatment of various ratios of irradiated butene-nitrogen dioxide mixture in air is shown in Table III. The concentrations and flows in the first experiment resulted from error in mechanical adjustments, but has been included since both products of interest, III and VI, were formed. Reduction of the butene-nitrogen dioxide ratio as well as absolute concentrations gave comparable amounts of III, but decreasing amounts of VI with the appearance of V. It may or may not be significant that irradiation of air alone, V was formed.

Although this series of experiments is incomplete, the observations invite speculation that nitrogen dioxide is serving as a radical scavanger. Conspicuously absent is the exposure of limonene to irradiated butene in air, the reduction of concentrations of butene and nitrogen dioxide by an order of magnitude as well as the unirradiated controls. Even at present, the data suggests the presence of singlet oxygen in some of the mixtures and indicates that the products merit further identification.

Quite apart from further identification of those producst which may arise by way of singlet oxygen, these experiments further indicate the diversity of effects with varying concentrations and ratios of hydrocarbon/nitrogen dioxide mixtures. It appears possible that limonene could serve to elucidate the diverse reactions which may

be occuring and thus the differing biochemical responses which have been observed.

SUMMARY

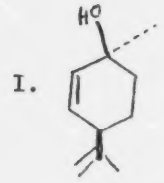
Preliminary experiments indicate that the turpene, d-limonene, can serve to differentiate the types of oxidizing reactants formed from photolysis of air pollution mixtures (smog); thus deliniating those air pollution mixtures which are of biological concern. In addition this will serve to elucidate the variables involved in the previous observed biochemical responses which, in turn, should assist in the development of valid dose-response relationships.

37<

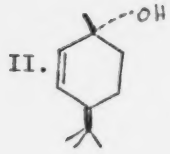
TABLE I

Isomeric Alcohols from Oxidation of

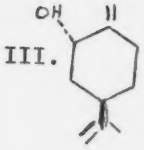
d-Limonene

I. 

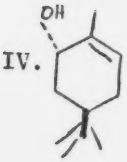
trans p-methadien-2, 8-ol-1

II. 

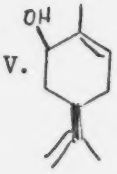
cis p-methadien-2, 8-ol-1

III. 

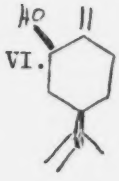
p-methadien-1(7) 8 trans-ol-2

IV. 

p-methadien-1, 8 trans-ol-6
Also known as trans carveol

V. 

p-methadien-1, 8 cis-ol-6
Also known as cis carveol

VI. 

p-methadien-1(7), 8 cis-ol-2

38<

TABLE II

Analysis of Products of Limonene

After Various Treatments

Treatment	None	H_2O_2/ClO	Air	Irradiated Air	Air-NO_2 Irradiated	Identification Based on
Total Count	163404	162739	151981	155990	161997	Retention Time
Time						
97-101	90393	91260	87014	86413	89609	limonene
160-164	----	847	496	359		
197		1024		762		
208	6226		6059		3164	
219		7628		2600		
234				1810		
300	276	---	----	152	170	
350	657	920	1673	----	1379	
371		954		429		
409	394	1069	----	1227	998	
430	---	---	364	763		
449	---	820			121	
452			616			
550		165	424	470		Carvone
580		229	1208		935	I
610		1347				II
704				1044	501	III
726		703				
768			493			IV
773					476	
791				788		V
1189					1134	VI

Experimental Conditions

NO_2 Concn. ppm			0.86
Flow Rate ml/min	120	120	120
Exposure Time, Hrs.	6	12	6

TABLE III

Effect of Exposure to

Synthetic Pollutant Mixtures

Total Count	175180	153786	160686	181776	Identification Based on Retention Time
Time					
91	89559	83257	87087	95695	Limonene
137	830	230	---	---	
183	3559	540	230	1539	
203	3564	624	---	10465	
225	---	6456	7062	---	
281	351	---	---	1228	
319	1427	509	232	2753	
375	919	386	1604	1841	
407	799	---	---	1591	
427	---	1559	1101	---	
460	356	895	485	---	
530	1275	1579	320	1191	
594	---	2783	2773	5112	Carvone
658	1113	---	---		I
745	868	1180	1311		II
821	---	581	799		III
1134	654	>	>	>	V
					VI

Experimental Conditions				
Butene Conc ppm	2000	50	50	33
NO_2 Conc ppm	7.3	0.5	5	6.6
Ratio	300:1	100:1	10:1	5:1
Flow Rate ml/min	60	120	120	120
Irradiation Time Hours	6	6	6	6

40<

REFERENCES

1. Estes, F. L., Karl O. M. Ferenczy. "Apparatus for Delivering Constant Concentrations of Components in Gas Mixtures," American Chemical Society Preprint, Div. of Water, Air, and Waste, Vol. 8, p. 86, Sept. 1968.

2. Foote, C. S., S. Wexler, A. Wataru and R. Higgins. "Chemistry of Singlet Oxygen IV Oxygenation with Hypochlorite-Hydrogen Peroxide," Journal Am. Chem. Soc., 90 975, 1968.

3. Foote, C. S., S. Wexler and W. Ando. "Chemistry of Singlet Oxygen III Product Selectivity," Tetrahedron Letters 46 4111, 1965.

4. Wexler, S. "Photoxygenation of Olefins. The Role of Singlet Molecular Oxygen." Dissertation. University of California, Los Angles, 1966.

41<